

**PATENT**#8  
Linda  
10/19/00**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE****In re Application of:**

Havenga, et al.

**Serial No.: 09/348,354****Filed: July 7, 1999****For: CHIMERIC ADENOVIRUSES****Examiner: Y. Connell****Group Art Unit: 1633****Attorney Docket No.: 4123US****DECLARATION UNDER 37 C.F.R. § 1.132**

I, MENZO HAVENGA, a citizen of the Netherlands, do hereby declare and say as follows:

That I received a degree in Biology / Biochemistry from the University of Nijmegen, in 1991; and a Doctor of Philosophy in Molecular Genetics from University of Leiden, in 1993.

That I am among the joint inventors of the referenced patent application, Chimeric Adenoviruses, and that the invention was the product of a collaboration between me and my co-inventors, Dr. Ronald Vogels, and Dr. Abraham Bout, both citizens of the Netherlands;

That I conducted (or worked directly with) the series of tests related to this Declaration in order to determine, evaluate, and compare the presence in humans of neutralizing antibodies against a panel of 44 human adenovirus serotypes.

That the series of tests related to this Declaration are demonstrative of a range of antigenicities for the various human adenovirus serotypes tested.

I hereby declare that the enclosed Figures 1-4 do correctly summarize the hereinafter

described materials, methods, procedures, and results.

I. A high throughput assay for the detection of neutralizing activity in human serum (see also the referenced patent application: *Chimeric Adenoviruses*, pp 55-60)

To enable screening of a large amount of human sera for the presence of neutralizing antibodies against a panel of 44 human adenovirus serotypes, an automated 96-well assay was developed.

A. *Human sera*

A panel of 100 individuals was selected. Volunteers (50% male, 50% female) were healthy individuals between ages 20 and 60 years old with no restriction for race. *Id.* at 55. All volunteers signed an informed consent form. People professionally involved in adenovirus research were excluded. *Id.*

Approximately 60 ml blood was drawn in dry tubes. *Id.* at 56. Within two hours after sampling, the blood was centrifuged at 2500 rpm for 10 minutes. *Id.* Approximately 30 ml serum was transferred to polypropylene tubes and stored frozen at -20° C until further use. *Id.*

Serum was thawed and heat-inactivated at 56° C for 10 minutes, then aliquoted to prevent repeated cycles of freeze/thawing. *Id.* Part of the serum was used to make five steps of twofold dilutions in medium (DMEM, Gibco BRL) in a quantity enough to fill out approximately 70 96-well plates. *Id.* Aliquots of undiluted and diluted sera were pipetted in deep well plates (96-well format) and using a programmed platemate dispensed in 100 µl aliquots into 96-well plates. *Id.* This way the plates were loaded with eight different sera *in duplo* (100 µl/well) of different dilutions ranging from 4x to 64x dilutions. *Id.* The last plates also contained four wells filled with 100 µl fetal calf serum as a negative control. *Id.* Plates were kept at -20° C until further use. *Id.*

B. *Preparation of human adenovirus stocks*

Prototypes of all known human adenoviruses were inoculated on T25 flasks seeded with PER.C6 cells (Fallaux *et al.*, 1998) and harvested upon full CPE. After freeze/thawing, 1-2 ml of the crude lysates were used to inoculate a T80 flask with PER.C6 (U.S. Patent 5,994,128), and virus was harvested at full CPE. *Id.* at 57. The time frame between inoculation and occurrence of CPE, as well as the amount of virus needed to re-infect a new culture, differed between serotypes.

Adenovirus stocks were prepared by freeze/thawing and used to inoculate 3-4 T175 cm<sup>2</sup> three-layer flasks with PER.C6 cells. *Id.* Upon occurrence of CPE, cells were harvested (by tapping the flask), pelleted, and virus was isolated and purified by a two-step CsCl gradient as follows. *Id.* Cell pellets were dissolved in 50 ml 10 mM NaPO<sub>4</sub> buffer (pH 7.2) and frozen at -20°C. After thawing at 37°C, 5.6 ml sodium deoxycholate (5% w/v) was added. *Id.* The solution was mixed gently and incubated for 5-15 minutes at 37°C to completely lyse the cells. *Id.* After homogenizing the solution, 1875 µl 1M MgCl<sub>2</sub> was added. *Id.* After the addition of 375 µl DNase (10 mg/ml) the solution was incubated for 30 minutes at 37°C. *Id.* Cell debris was removed by centrifugation at 1880xg for 30 minutes at RT without brake. *Id.* The supernatant was subsequently purified from proteins by extraction with FREON (3x). *Id.* The cleared supernatant was loaded on a 1M Tris/HCl buffered cesium chloride block gradient (range: 1.2/1.4 g/ml) and centrifuged at 21000 rpm for 2.5 hours at 10°C. *Id.* The virus band was isolated, followed by a second purification using a 1M Tris/HCl buffered continuous gradient (of 1.33 g/ml of cesium chloride). *Id.* The virus was then centrifuged for 17 hours at 55000 rpm at 10°C. *Id.* The virus band was then isolated and sucrose (50 % w/v) was added to a final concentration of 1%. *Id.* at 58. Excess cesium chloride was removed by dialysis (three times 1 hr at RT) in dialysis slides (Slide-a-lizer, cut off 10000 kDa, Pierce, USA) against 1.5 liter PBS supplemented with CaCl<sub>2</sub> (0.9 mM), MgCl<sub>2</sub> (0.5mM) and an increasing concentration of sucrose (1, 2, 5%). *Id.* After dialysis, the virus was removed from the slide-a-lizer, aliquoted in portions of 25 and 100 µl, then stored at -85°C.

To determine the number of virus particles per milliliter, 50 µl of the virus batch was run on a high-pressure liquid chromatograph (HPLC) as described by Shabram et al (1997). *Id.* Viruses were eluted using a NaCl gradient ranging from 0 to 600 mM. *Id.* The NaCl concentration by which the viruses were eluted differed significantly among serotypes. *Id.*

Most human adenoviruses replicated well on PER.C6 cells with a few exceptions. *Id.* Adenovirus types 8 and 40 were grown on 911-E4 cells (He *et al.*, 1998). *Id.* Purified stocks contained between 5x10<sup>10</sup> and 5x10<sup>12</sup> virus particles/ml (VP/ml; see table I). *Id.*

### *C. Titration of purified human adenovirus stocks*

To determine the amount of virus necessary to obtain full CPE in five days, adenoviruses were titrated on PER.C6 cells across the length of the neutralization assay. *Id.* Hereto, 100µl medium was

dispensed into each well of 96-well plates. *Id.* 25  $\mu$ l of adenovirus stocks, pre-diluted  $10^4$ ,  $10^5$ ,  $10^6$  or  $10^7$  times, were added to column 2 of a 96-well plate and mixed by pipetting up and down 10 times. *Id.* Then 25  $\mu$ l was brought from column 2 to column 3 and again mixed. *Id.* This column-to-column sequence was repeated until reaching column 11, after which 25  $\mu$ l from column 11 was discarded. *Id.* This way, serial dilutions in steps of 5 were obtained starting off from a pre-diluted stock.  $3 \times 10^4$  PER.C6 cells (ECACC deposit number 96022940) were then added in a 100  $\mu$ l volume and the plates were incubated at 37 °C, 5% CO<sub>2</sub> for five or six days. CPE was monitored microscopically. *Id.* The method of Reed and Muensch was used to calculate the cell culture-inhibiting dose 50% (CCID50). *Id.* at 59.

In parallel, to distinguish between proceeding and full CPE, identical plates were set up for analysis using the MTT assay (Promega). *Id.* In this assay living cells were quantified by colorimetric staining. *Id.* Hereto, 20  $\mu$ l MTT (7.5 mgr/ml in PBS) were added to the wells and incubated at 37 °C, 5% CO<sub>2</sub> for two hours. *Id.* The supernatant was removed and 100  $\mu$ l of a 20:1 isopropanol/triton-X100 solution was added to the wells. *Id.* The plates were then put on a 96-well shaker for 3-5 minutes to solubilize the precipitated staining. Absorbance was measured at 540 nm and at 690 nm (background). *Id.*

#### *D. Neutralization assay*

96-well plates with diluted human serum samples were thawed at 37 °C, 5% CO<sub>2</sub>. *Id.* Adenovirus stocks (diluted to 200 CCID50 per 50  $\mu$ l) were prepared, and 50  $\mu$ l aliquots were added to columns 1-11 of the plates containing serum. *Id.* Plates were incubated for 1 hour at 37°C, 5% CO<sub>2</sub>. *Id.* Then 50  $\mu$ l PER.C6 cells at  $6 \times 10^5$ /ml were dispensed in all wells and incubated for 1 day at 37 °C, 5% CO<sub>2</sub>. *Id.* Supernatant was removed using fresh pipette tips for each row, and 200  $\mu$ l fresh medium was added to all wells to avoid toxic effects of the serum. *Id.* Plates were incubated for another 4 days at 37 °C, 5% CO<sub>2</sub>.

In addition, parallel control plates were set up *in duplo* with diluted positive control sera generated in rabbits (and specific for each serotype) to be tested in rows A and B and with negative control serum (FCS) in rows C and D. *Id.* Also, in each of the rows E-H a titration was performed, as described with steps of five times dilutions, starting with 200 CCID50 of each virus to be tested. *Id.* On day 5, one of the control plates was analyzed both microscopically and with the MTT assay.

*Id.* The experimental titer was calculated from the control titration plate observed microscopically. *Id.* at 60. If CPE was found to be complete, *i.e.* the first dilution in the control titration experiment analyzed by MTT shows clear cell death, all assay plates were processed. *Id.* If not, the assay was allowed to proceed for one or more days until full CPE was apparent, after which all plates were processed. *Id.* In most cases, the assay was terminated at day 5. *Id.* For Ad1, 5, 33, 39, 42 and 43 the assay was left for six days and for Ad2 for eight days. *Id.* A serum sample is regarded as “non-neutralizing” when, at the highest serum concentration, a maximum protection of 40% is seen compared to controls without serum. *Id.*

### *1. Figure 1: All serotypes compared*

The results of the analysis of 44 prototype adenoviruses against serum from 100 healthy volunteers are shown in FIG. 1. *Cf. id.* at 18, lines 20-26; *Cf. id.* at 20, lines 11-18. As expected, the percentage of serum samples that contained neutralizing antibodies to Ad2 and Ad5 was very high. This was also true for most of the lower numbered adenoviruses. Surprisingly, none of the serum samples contained neutralizing antibodies to Ad35. Also, the number of individuals with neutralizing antibody titers to the serotypes 26, 34 and 48 was very low. Therefore, recombinant E1-deleted adenoviruses based on Ad35 or one of the other above mentioned serotypes have an important advantage compared to recombinant vectors based on Ad5 with respect to clearance of the viruses by neutralizing antibodies.

Also, Ad5-based vectors that have (parts of) the capsid proteins involved in immunogenic response of the host replaced by the corresponding (parts of) the capsid proteins of Ad35 or one of the other serotypes will be less, or even not, neutralized by the vast majority of human sera.

### *2. Figure 2: Log ratio VP/CCID50 versus % neutralization*

The VP/CCID50 ratio calculated from the virus particles per ml and the CCID50 obtained for each virus in the experiments was highly variable, and ranged from 0.4 to 5 log. This is probably caused by different infection efficiencies of PER.C6 cells and by differences in replication efficiency of the viruses. Also, differences in batch qualities may play a role. For example, a high VP/CCID50 ratio means that more viruses were put in the wells to obtain CPE in 5 days. As a consequence, the outcome of the neutralization study might be biased since more (inactive) virus particles could

shield the antibodies. To check whether this phenomenon had taken place, the VP/CCID50 ratio was plotted against the percentage of serum samples found positive in the assay (FIG. 4). The graph clearly shows that there is no negative correlation between the amount of viruses in the assay and neutralization in serum.

*E. The prevalence of neutralizing activity (NA) to Ad35 is low in human sera from different geographic locations*

In Part A of this Declaration, the analysis of neutralizing activity ("NA") in human sera was described for one location: Belgium. Strikingly, of a panel of 44 adenovirus serotypes tested, one serotype, Ad35, was not neutralized in any of the 100 sera assayed. In addition, a few serotypes, Ad26, Ad34 and Ad48 were found to be neutralized in 8%, or less, of the sera tested. This analysis was further extended to other serotypes of adenovirus not previously tested and, using a selection of serotypes from the first screen, was also extended to sera from different geographic locations. Hereto, adenoviruses were propagated, purified and tested for neutralization in the CPE-inhibition assay as described in Part A of this Declaration. Using the sera from the same batch as in Part A, adenovirus serotypes 7B, 11, 14, 18 and 44/1876 were tested for neutralization. These viruses were found to be neutralized in, respectively, 59, 13, 30, 98 and 54 % of the sera. Thus, of this series, Ad11 is neutralized with a relatively low frequency (please see Figures 1, 3, and 4). *Cf. id.* at 18, lines 20-26; *Cf. id.* at 20, lines 11-18.

Since it is known that the frequency of isolation of adenovirus serotypes from human tissue as well as the prevalence of NA to adenovirus serotypes may differ according to locale, we tested a selection of the adenovirus serotypes against sera from different geographic locations. To facilitate this, human sera were obtained from two additional places in Europe (Bristol, UK and Leiden, NL) and from two places in the United States (Stanford, CA and Great Neck, NY). Figure 3 represents the first screen of neutralization data, comparing the UK and Belgium only. Figure 4 represents the second screen of neutralization data, comparing the UK, Belgium, both US locations, and NL. *Cf. id.* at 18, lines 20-26; *Cf. id.* at 20, lines 11-18.

*1. Figure 2: Belgium and UK compared*

Adenoviruses that were found to be neutralized in 20% or less of the sera in the first screen, as well as Ad2, Ad5, Ad27, Ad30, Ad38, Ad43, were tested for neutralization in sera from the UK (see

Figure 3). Adenovirus serotypes 2 and 5 were again neutralized in a high percentage of human sera. Furthermore, some of the serotypes that were neutralized in a low percentage of sera in the first screen were neutralized in a higher percentage of sera from the UK (e.g. Ad26 (7% vs. 30%), Ad28 (13% vs. 50%), Ad34 (5% vs. 27%) and Ad48 (8% vs. 32%)). Neutralizing activity against Ad11 and Ad49 that were found in a relatively low percentage of sera in the first screen, were found in an even lower percentage of sera in this second screen (13% vs. 5% and 20% vs. 11% respectively). Serotype Ad35 that was not neutralized in any of the Belgium sera in the first screen, was found to be neutralized in a low percentage (8%) of sera from the UK. The prevalence of NA in human sera from the UK is the lowest to serotypes Ad11 and Ad35. *Cf. id.* at 18, lines 20-26; *Cf. id.* at 20, lines 11-18.

## 2. Figure 4: Belgium, UK, US(1-2), and NL

For further analysis, sera were obtained from two locations in the US (Stanford, CA and Great Neck, NY) and from The Netherlands (Leiden). FIG. 3 presents an overview of data obtained with these sera and the previous data. Not all viruses were tested in all sera, except for Ad5, Ad11 and Ad35.

The overall conclusion from this comprehensive screen of human sera is that the prevalence of neutralizing activity to Ad35 is the lowest of all serotypes throughout the western countries: on average 7% of the human sera contain neutralizing activity (5 different locations). Another B-group adenovirus, Ad11, is also neutralized in a low percentage of human sera (average 11% in sera from 5 different locations). Adenovirus type 5 is neutralized in 56% of the human sera obtained from 5 different locations. Although not tested in all sera, D-group serotype 49 is also neutralized with relatively low frequency in samples from Europe and from one location of the US (average 14%). *Cf. id.* at 18, lines 20-26; *Cf. id.* at 20, lines 11-18.

## F. Discussion

In the herein described neutralization experiments, a serum is judged non-neutralizing when, in the well with the highest serum concentration, the maximum protection of CPE is 40% compared to the controls without serum. The protection is calculated as follows:

$$\% \text{ protection} = \frac{\text{OD corresponding well} - \text{OD virus control}}{\text{OD non-infected control} - \text{OD virus control}} \times 100 \%$$

As described in Part A of this Declaration, the serum is plated in five different dilutions ranging from 4x to 64x diluted. Therefore, it is possible to distinguish between low titers (i.e., neutralization only in the highest serum concentrations) and high titers of NA (i.e., also neutralization in wells with the lowest serum concentration). *Id.* at 54-55. Of the human sera used in our screen that were found to contain neutralizing activity to Ad5, 70% turned out to have high titers; whereas, of the sera that contained NA to Ad35, only 15% had high titers. Of the sera that were positive for NA to Ad11 only 8% had high titers. For Ad49, this was 5%. Therefore, not only is the frequency of NA to Ad35, Ad11 and Ad49 much lower as compared to Ad5, but of the sera that do contain NA to these viruses, the vast majority has low titers. Adenoviral vectors based on Ad11, Ad35 or Ad49 have therefore a clear advantage over Ad5 based vectors when used as gene therapy vehicles or vaccination vectors in vivo or in any application where infection efficiency is hampered by neutralizing activity.

## II. Declaration

I, Menzo Havenga, do hereby declare under penalty of perjury under the laws of the United States of America that the foregoing is true and correct to the best of my information and belief.

M. Havenga  
Menzo Havenga

Executed this 01 day of August, 2000.

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**Figure 1**  
**% of human sera with neutralising capacity for human adenovirus (n=100)**

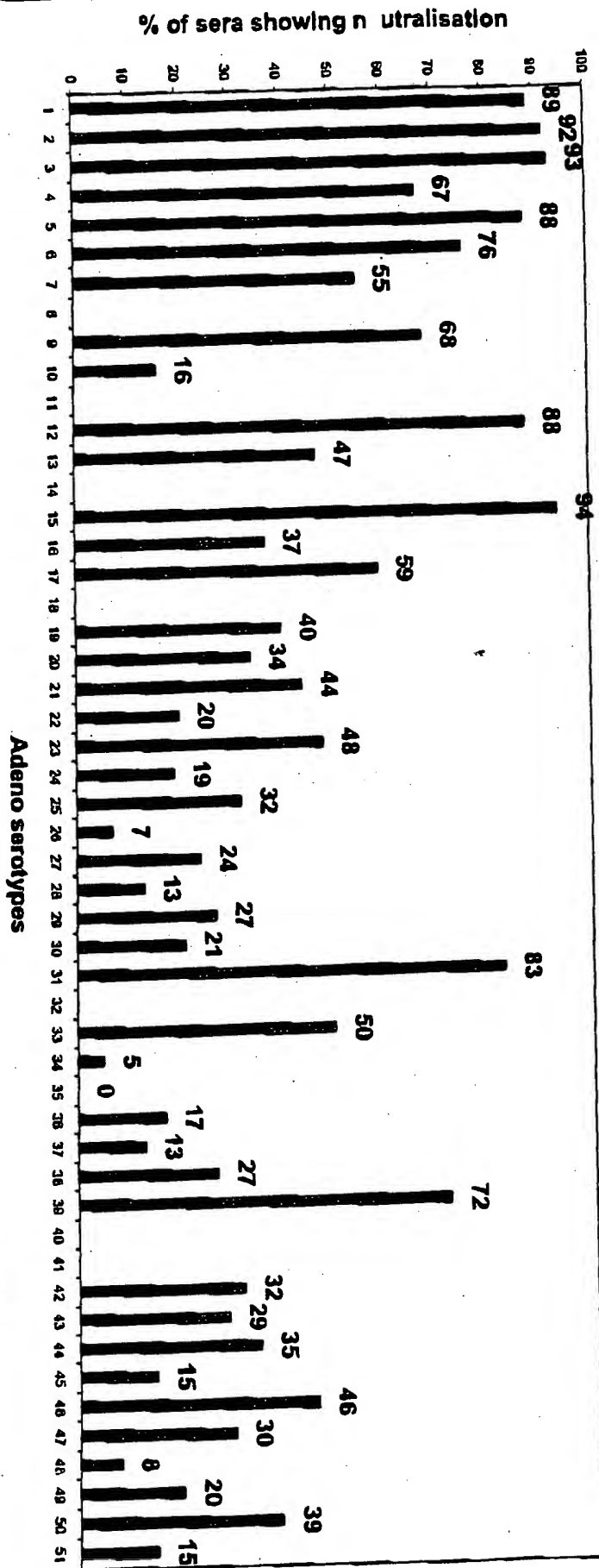
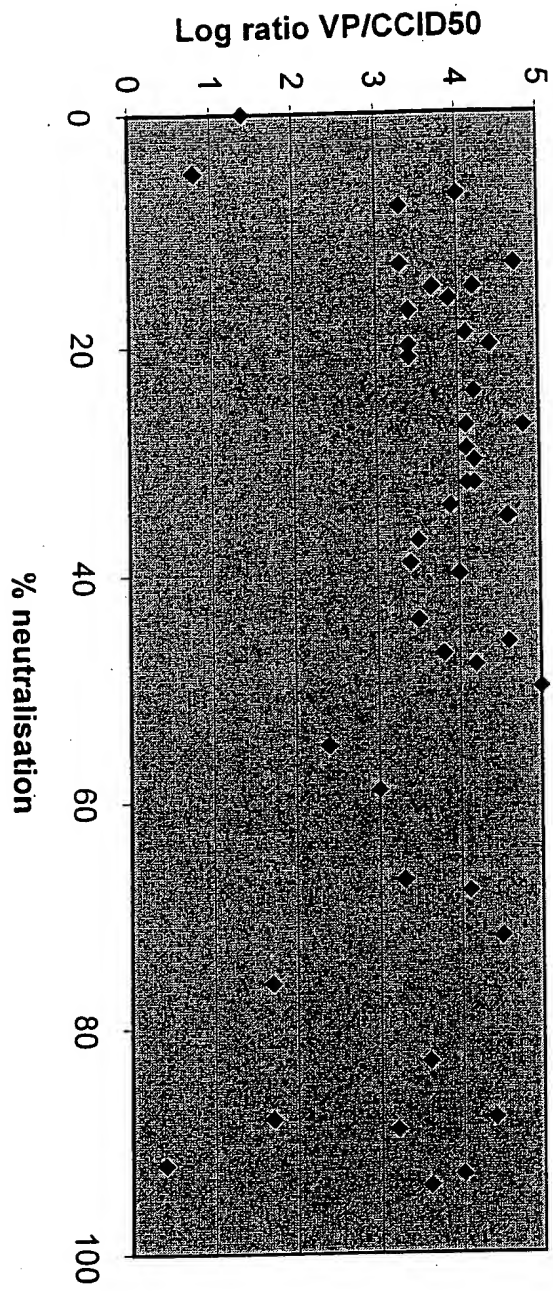
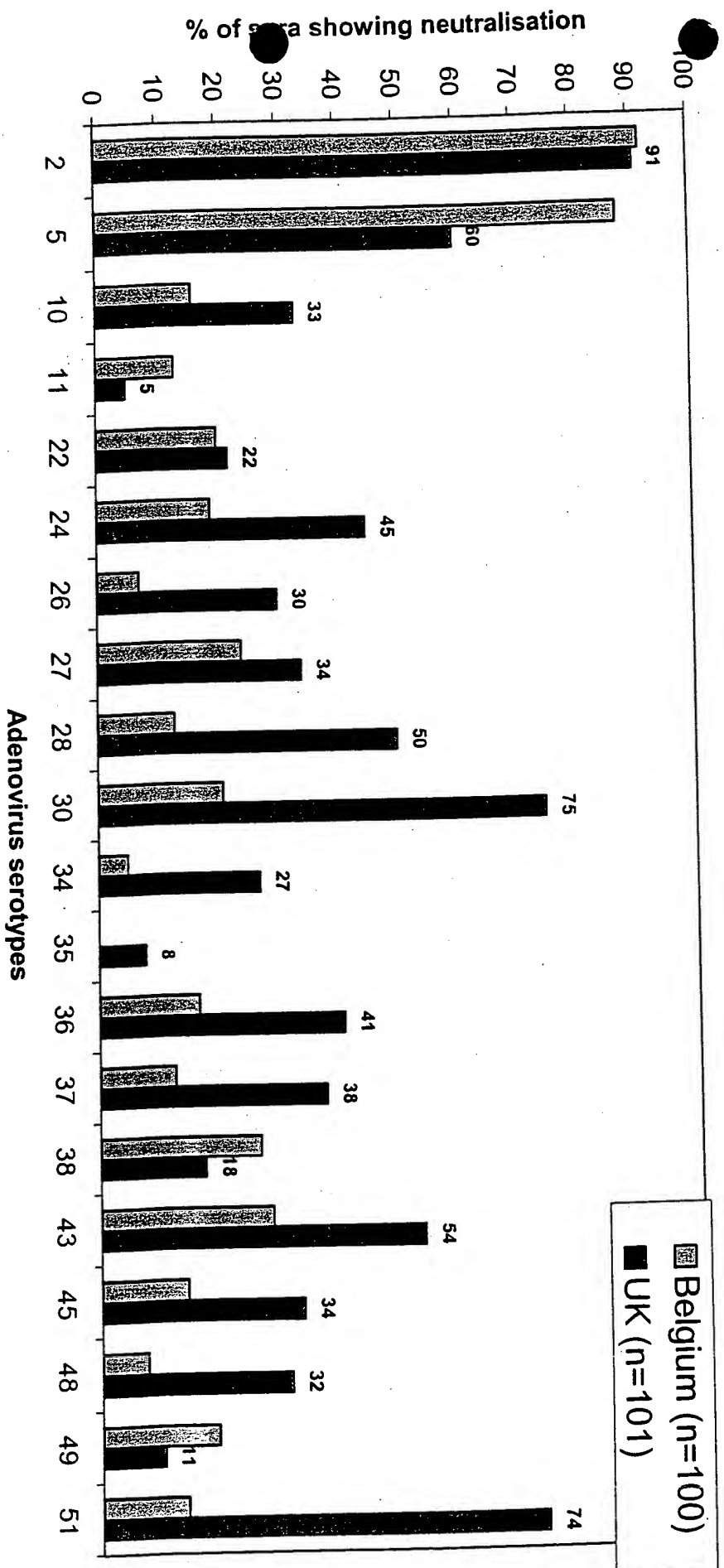


Figure 2



**Figure 3**  
**Neutralisation in human sera**



**Figure 4**  
**Neutralisation in human sera from different geographic locations**

